

# In vivo antimalarial activity of the crude leaf extract of *Combretum molle* against *Plasmodium berghei* in mice

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## Research

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**Abstract**

**Background:** Malaria is an infectious, hematologic disease causing death and illness in children and adults, especially in tropical countries. The aim of this study was to evaluate the antimalarial activity of *Combretum molle* extract *in vivo* assays against *Plasmodium berghei* in Swiss albino mice.

**Methods:** *Plasmodium berghei* a rodent malaria parasite was inoculated to healthy Swiss Albino mice age 6–8 weeks either sex, weight 20–33g. 100, 200 and 400mg/kg/day of Crude methanolic extract of *Combretum molle* were administered. Parameters such as Percent parasitemia, body weight, Body temperature, packed cell volume and survival time were then determined using standard tests. Data were analyzed using one-way ANOVA followed by the Post hoc Tukey HSD test with SPSS software version 24.0 and  $P \leq 0.05$  considered as statistically significant.

**Results:** Chemosuppressive effect exerted by the crude extract ranged between 27-68%. The curative effect of the crude extract was in the range of 25-49% and prophylactic effect of the crude extract was in the range of 51–76.2%. The maximum effect in all tests on Chemosuppressive, curative, Prophylactic, prevention of weight loss, body temperature and packed cell volume and an increase in mean survival time was observed at higher doses of the crude extract.

**Conclusion:** From the present study it can be concluded that the crude extract of *Combretum molle* leaves has been shown promising antimalarial activity. This finding supports the traditional use of the plant for the treatment of malaria in Ethiopia. Thus, it could be considered as a potential source to develop safe, effective and affordable antimalarial agent.

**Key Words:** Antimalarial, *Combretum molle*, crude extract, *Plasmodium berghei*, sub-acute toxicity

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### 63 **Background**

64 Malaria is an infectious, hematologic disease <sup>[1]</sup> causing death and illness in  
65 children and adults <sup>[2]</sup>. Malaria is caused by single-cell Plasmodium species that  
66 invade, grow and replicate in red blood cells in a cyclic pattern which makes it such  
67 a deadly disease<sup>[3, 4]</sup>. Malaria control requires prevention, primarily vector control,  
68 and prompts treatment with effective antimalarial drugs <sup>[5]</sup>. Malaria is among the  
69 most devastating and widespread tropical parasitic diseases in which most prevalent  
70 in developing countries <sup>[6]</sup>. Antimalarial drug resistance results in a global  
71 resurgence of malaria making a major threat to malaria control.<sup>[7]</sup>

72 From the World Health Organization (WHO) 2018 malaria report 219 million cases  
73 of malaria from 90 countries. The WHO African region with 200 million cases  
74 (92%) in 2017, especially Sub-Saharan Africa suffers by far the greatest malaria  
75 burden worldwide. Almost 93% of all deaths due to malaria in 2017 were from  
76 Africa <sup>[8]</sup>.

77 Seventy percent of malaria caused deaths occurring among the under five-year age  
78 group. Nigeria, the Democratic Republic of the Congo, Burkina Faso and India  
79 accounted for 47% of all malaria deaths globally <sup>[9]</sup>. *P. falciparum* species are  
80 dominant in Africa and *P.vivax* infection is in Southeast Asia and South America  
81 <sup>[10]</sup>. In pregnant, women *P. falciparum* causes infections because of parasite's  
82 ability to massively sequester in the placenta and compromised immune status,  
83 mainly in the first and second trimesters of pregnancy <sup>[11]</sup>.

84 About 60% of the Ethiopian population is at risk of malaria with major  
85 transmission seasons from June to December and remain responsible for a massive  
86 burden of disease and death <sup>[12, 13]</sup>.

### 87 **The experimental plant**

88 The Combretaceae is a large family of herbs, shrubs, and trees, comprising about 20  
89 genera and 600 species with tropical distribution around the globe and centers of  
90 diversity in Africa and Asia. Simple hairs of indumentum, multicellular or  
91 glandular hairs secreting calcium oxalate and forming scales or present beneath  
92 cuticle and making leaf blade surface verruculose and sometimes translucent

93 dotted. Leaves (opposite, sub opposite, whorled, spiraled, or alternate, usually  
94 petiolate), flowers are unisexual or bisexual and the seed is usually one-seeded and  
95 flattened <sup>[14]</sup>.

96 Combretum is the largest and most widespread genus of Combretaceae which  
97 comprises approximately 200-250 species. *Combretum molle* is a shrub or small,  
98 graceful, deciduous tree, 3 - 13 m high, with a crooked or lean trunk, occasionally  
99 swollen at the base, and up to 30 cm in diameter. *Combretum molle*  
100 (R.Br.ex.G.Don) Engl and Diels are indigenous to Africa and are one of the  
101 important medicinal species of the family <sup>[15]</sup>. Different parts of the *Combretum*  
102 *molle* are widely used as a medicinal plant to treat various diseases such as  
103 parasitic, protozoan and other infectious diseases <sup>[16]</sup>.

104 The Fresh leaf is boiled in water and the decoction is drunk by a cup of tea which  
105 was studied from Delanta (Amhara region), and Amaro District (Southern Nations  
106 Nationalities People region). Probably leaves are the center of intermediary  
107 metabolism leading to biologically active secondary metabolites <sup>[17, 18]</sup>. The *in vitro*  
108 cell culture (K562S human monocyte infected with *P. falciparum* W2) study had  
109 shown activity with an IC<sub>50</sub> value of 5.7 or 7.9µg/mL, respectively from 80%  
110 methanol extract of leaves. Ethanol 90% extract of leaves, root bark or stem bark *in*  
111 *vitro* cell culture with *P. falciparum* K1 showed activity with an IC<sub>50</sub> value of 4.0  
112 µg/mL<sup>[19]</sup>.

113 Stem bark *in vitro* cell culture (Trophozoites of *P. falciparum*) of acetone fraction  
114 showed activity with IC<sub>50</sub> value of 8.17µg/mL. MeOH extract of dried stem *in vitro*  
115 cell culture with *Plasmodium falciparum* also showed activity with an IC<sub>50</sub> value of  
116 1.25 µg/MI <sup>[20, 21]</sup>.

## 117 **Methods**

### 118 **Plant Material**

119 Fresh leaves of *Combretum molle* were collected from “Tara Gedam”, Libo  
120 Kemkem District, South Gondar Zone, Amhara region 85 Kilometer away from  
121 Gondar city on December 13, 2018. The collected plant material was wrapped and  
122 covered with plastic sheets during transportation. The specimen of the plant was  
123 authenticated by a botanist, in the biology department, University of Gondar and

124 was deposited in the herbarium with a voucher specimen (No 001MAS) for future  
125 reference.

### 126 **Preparations of crude methanolic extract**

127 The leaves of *C.molle* were washed with tap water gently to eliminate any dirt and  
128 dried at ambient temperature, protected from light, reduced to an appropriate size  
129 by hand compression and stored at ambient temperature until extraction. 1500g of  
130 leaf powder of *C.molle* was weighed with electric balance and transferred to the  
131 Erlenmeyer flasks and 80% methanol (one gram of *C.molle* powdered leaf to ten  
132 milliliters of 80% methanol ratio) was added and agitated in between. After 72  
133 hours of maceration extraction was performed using thick layers of 40 mesh gauze  
134 and then filtered with Whatman filter paper no1 two times and 2<sup>nd</sup> extraction was  
135 repeated after 72 hours of maceration and the 3<sup>rd</sup> extraction was repeated another  
136 72 hours later (with a total of nine days). The extracts were combined and  
137 concentrated by the dry oven at 40<sup>0</sup>C and the yield was determined and found to be  
138 287g (19.13%) and stored in a refrigerator at 4<sup>0</sup>C until use <sup>[22]</sup>.

### 139 **Phytochemical screening**

140 Secondary metabolites are classes of compounds that are known to show a curative  
141 activity against several ailments in humans and then could explain the traditional  
142 use of medicinal plants for the treatment of some illnesses. Complex structural  
143 molecules such as alkaloids, Phenolic compounds, anthraquinones, resins,  
144 saponins, steroids, and terpenoids are found in a more restricted distribution than  
145 primary metabolites. They are not indispensable for the plant that contains them; at  
146 least their metabolic functions have not been discovered yet<sup>[23]</sup>. Standard qualitative  
147 tests were employed to detect secondary metabolites <sup>[24 - 31]</sup>.

### 148 **Animals and parasite**

149 Swiss albino mice weighing between 20 - 33g, 6 – 8 weeks of age and either sex  
150 were purchased from the Ethiopian public health institute (EPHI) Addis Ababa.  
151 The animals were kept in cages and housed in a standard animal house under a  
152 natural 12/12h light-dark cycle at room temperature and maintained on a standard  
153 pellet diet and water ad libitum. Before the experiment, they were acclimatized to  
154 the test environment for one week. The care and handling of mice were according

155 to international guidelines for the use and maintenance of experimental animals [32,  
156 33].

157 Chloroquine-sensitive malaria, *P. berghei* was obtained from EPHI in the  
158 traditional medicine research department Addis Ababa and transported by infecting  
159 the donor mice and maintained the parasite up to the main procedure of the study.

#### 160 **Acute toxicity test**

161 Five mice weighing between 20 - 28 grams, 6-8weeks of age female sex were kept for one  
162 week prior to dosing to acclimatize. One mouse was randomly taken for sighting study and  
163 food but not water was withheld for 4 hours. Following the period of fasting, the mouse  
164 was weighed and the crude methanolic extract of *C. molle* leaf 2000mg/kg body weight  
165 was administered by the oral route. Strict observation within 30minutes and periodically  
166 during the first 24 hours, with special attention given during the first 4 hours and daily  
167 thereafter, for a total of 14 days. Changes in skin and fur, eyes, mucous membranes,  
168 respiratory, autonomic and central nervous system changes, somatomotor activity and  
169 behavior patterns were observed. Tremors, convulsions, salivation, diarrhea, lethargy, sleep  
170 and coma were evaluated. But the mouse in the sighting test did not show any sign of  
171 toxicity for four-days and the main toxicity doses of the remaining 4 mice for methanolic  
172 crude extract were given and followed as usual for 14 days [32, 34].

#### 173 **Subacute toxicity test**

174 The subacute toxicity study was conducted on 24 healthy adult female mice divided  
175 into four groups of six each. The control group received solvent; the other three  
176 groups were given 100, 200 and 400mg/kg/day of *C. molle* crude extract orally for  
177 28 days. The mice were observed every two hours for toxic signs and their weights  
178 were measured weekly for five weeks. On the 28<sup>th</sup> day, mice were anesthetized  
179 with ketamine and sacrificed. Their Liver and kidneys were removed and weighed  
180 [32, 35].

#### 181 **Tissue processing**

182 Immediately removed liver and kidneys were placed in 10% formalin with  
183 phosphate-buffered saline solution overnight at room temperature. After overnight  
184 fixation, the tissues were dehydrated with increased concentration of alcohol: 70%,  
185 80%, 90% for five and half hour, then cleared with xylene for seven hours. Finally,  
186 the tissues were impregnated for six hours. Then, tissue blocks were prepared by

187 embedding with paraffin wax in a square metal plate and labeled. Tissue blocks  
188 were sectioned with a thickness of 5µm using a rotary microtome. Strips of sections  
189 were gently lowered into the surface of a warm water bath at 40°C for sections to  
190 be floated, then mounted with coated egg albumin slides and the slides containing  
191 paraffin wax within a slide holder were placed in an oven with a temperature of  
192 60°C for 10-15 minutes until they become free of wax.

193 Sectioned tissues were placed in xylene to deparaffinize and hydrated with  
194 decreasing alcohol concentrations, then stained with Hematoxyline for 5 minutes  
195 and counterstained by using eosin for one minute. After washing in tap water, the  
196 sections were dehydrated with increased alcohol concentration and cleared with  
197 xylene.

198 Finally sections were mounted on microscopic slides by using Canada balsam  
199 mount and cover slips and examined the tissue under the compound light  
200 microscope with different objectives were taken a photo automatically with  
201 magnification of x40 [36-38].

#### 202 **Pharmacologic screening (4-day Suppressive test)**

203 *In vivo* evaluation of methanolic leaf crude extract of *C. molle* antimalarial activity  
204 on rodent malaria parasite *P.berghei*. Donor *P. berghei* infected Swiss albino mice  
205 (parasitemia of approximately 30%) were sacrificed by decapitation and, then  
206 blood was collected through cardiac puncture with a sterile disposable needle and  
207 syringe. The blood was diluted with sterile sodium chloride 0.9% in water in such a  
208 way that 0.2ml of blood contained about 10<sup>7</sup> infected RBCs and mice were infected  
209 by 0.2ml blood suspension intraperitoneally which was expected to produce  
210 steadily rising consistent infection of the required intensity in mice [35]. Then Mice  
211 were randomly divided into five groups of six each, weighed, and maintained in a  
212 standard diet. The doses of crude extract were adjusted from the safest dose of  
213 acute-toxicity study and were 5%, 10%, and 20% (i.e. 100,200 and 400mg/kg) once  
214 daily for 4 days. The fourth group received chloroquine base 25mg/kg (positive  
215 control) and the fifth group was received solvent which contains 3ml of 96%  
216 ethanol + 7ml of Tween 80 + 90ml of distilled water (negative control)[39, 40] and



217 administered by oral route using oral gavage, starting from 3 hours of parasite  
218 inoculation.

### 219 **Determination of parasitemia**

220 On day 4 (96 hours) post-infection a drop of blood was collected from the mice by  
221 vein section of the tail and transferred onto the edge of a microscopic slide and  
222 drawn evenly across the second slide to make a thin blood film and allowed to dry  
223 at room temperature, fixed with methanol, stained with 10% Giemsa stain for 15  
224 minutes. Slides were viewed using light microscopy with oil immersion (1000 x  
225 magnification). Percentage parasitemia was determined by counting the number of  
226 PRBCs out of 200 RBCs in random fields of the microscope. Five fields of  
227 approximately 200 cells counted and the parasitemia was calculated as the  
228 percentage of the total RBCs containing PRBCs [41, 42]. The body weight, body  
229 temperature, packed cell volume and mean survival time were determined for each  
230 group arithmetically [40].

231 Percent parasitemia was calculated according to the following formula

$$232 \quad \% \text{ paristemia} = \frac{\text{Total number of PRBC}}{\text{Total number of RBC}} \times 100 \quad \text{Where:- PRBC: Parasitized red blood cells}$$

233 RBC: red blood cells

234 Average percentage of parasitemia suppression was calculated as follows:

$$235 \quad \text{Average \% of paristemia supression} = \frac{\text{Av. \% of parasit.in control} - \text{Av. \% of parasitemia in test}}{\text{Av. \% of parasitemia in control}} \times 100$$

236

Av = average

### 237 **Rane's (Curative) test**

238 Evaluation of the curative potential of methanolic leaf crude extract of *C. molle* was  
239 carried out according to the method described by curative methods [43]. On Day 0,  
240 standard inocula of about  $10^7$  infected erythrocytes were inoculated in mice  
241 intraperitoneally and randomly divided into their respective groups weighed and  
242 maintained in a standard diet. Seventy-two hours later they were dosed Crude  
243 methanolic extract at 100,200 and 400mg/kg/day, Chloroquine base 25mg/kg/day  
244 and solvent which were prepared from 3ml of 96% ethanol + 7ml of Tween 80 +  
245 90ml of distilled water once daily for five days. Giemsa stained thin blood film  
246 was prepared from the tail of each mouse daily for 5 days (day 3,4,5,6 & 7) to

247 monitor the parasitemia level. The body weight, body temperature, packed cell  
248 volume and mean survival time were determined for each group arithmetically <sup>[40]</sup>.

#### 249 **The Repository (Prophylactic) Test**

250 In prophylactic test prior to infection, mice in the test groups (three groups with  
251 100, 200 and 400mg/kg of crude extract) were administered once daily orally for  
252 three days. The remaining two groups were administered with the standard drug  
253 (chloroquine base 25 mg/kg) and vehicle (solvent 10ml/kg/day). On the fourth day,  
254 a standard inoculum of *Plasmodium berghei* containing  $\sim 1 \times 10^7$  infected  
255 erythrocytes was administered intraperitoneally to each mouse. On the seventh day  
256 of infection (seventy-two hours later), thin blood smears (Giemsa stained) were  
257 prepared from the tail of each mouse by vein section. Percentage parasitemia as  
258 well as the chemo suppression, body weight, body temperature, packed cell volume  
259 and mean survival time were determined for each group arithmetically <sup>[43]</sup>.

#### 260 **Measurement of Packed Cell Volume (PCV)**

261 Packed cell volume (PCV) was measured to predict the effectiveness of the test  
262 extract and fractions in preventing hemolysis resulting from increasing parasitemia  
263 associated with malaria. Heparinized capillary tubes were used for the collection of  
264 blood from the tail of each mouse. The capillary tubes were filled with blood up to  
265 three-fourth of their volume and sealed at the dry end with sealing clay. The tubes  
266 were then placed in a micro-hematocrit centrifuge, with the sealed end outwards  
267 and centrifuged for 5 min at 12,000 rpm. The tubes were taken out of the centrifuge  
268 and PCV was determined using a standard Micro-Hematocrit Reader <sup>[44, 45]</sup>.

#### 269 **Monitoring of body weight and temperature changes**

270 In Peter's test the bodyweight was measured one hour before parasite infection on  
271 D0 and at the end of D3 after treatment completion, while the rectal temperature  
272 was measured 1 hour before and 3 hours after parasite infection and then  
273 consecutively for 4 days to monitor the consequence of plant extracts on mice's  
274 body temperature. For Rane's test, the body weight and temperature were measured  
275 three hours before infection on day 0 (D0) and consecutively for five during the  
276 treatment period to establish the effect of plant drug on malarial mice. For the  
277 prophylactic test, the body weight and temperature were measured three hours

278 before infection on day 0 (D0) and consecutively from D4–D7 after infection  
279 period to establish the effect of plant extract on *P. bergeri* infected mice [46].

#### 280 **Data quality control**

281 The quality of data was assured by using randomization during the grouping of  
282 experimental animals. Unique codes were used for all frosted microscopic slides  
283 during the preparation of blood smears. Parasitized red blood cells were counted  
284 blindly by laboratory technologist (Medical laboratory technologist, MSc in  
285 medical parasitology).

#### 286 **Data analysis**

287 The results of the study were expressed as the mean  $\pm$  standard error of the mean  
288 (M  $\pm$  SEM). Comparison of parasitemia and statistical significance was determined  
289 by one-way ANOVA (a repeated measure of analysis of variance) descriptive  
290 statistics followed by Post Hoc tests for multiple comparisons of Tukey HSD and  
291 paired t-test was employed to test significance of the difference between initial and  
292 final results within the same and in between groups using SPSS for window  
293 (Version 24.0) statistical package. All data were analyzed at a 95% confidence  
294 interval ( $\alpha = 0.05$ ) was considered statistically significant [40].

### 295 **Results**

#### 296 **Phytochemical screening**

297 The phytochemical screening of 80% methanol crude extract of *Combretum molle*  
298 indicated the presence of alkaloids, cardiac glycosides, flavonoids, phenols,  
299 saponins, tannins, and terpenoids.

#### 300 **Acute oral toxicity test**

301 The acute oral toxicity study showed that no death and any sign of toxicity to mice  
302 with an oral dose of 2000mg/kg of the crude extract of *C. molle* observed for two  
303 weeks. Here it could be deduced that the lethal dose fifty (LD<sub>50</sub>) was above  
304 2000mg/kg per body weight. The bioactive constituents of the plant are safe for use  
305 even though it is difficult to extrapolate the result to humans.

#### 306 **Subacute toxicity study**

307 The crude extract of *C. molle* was administered for consecutive 28 days at doses of  
308 100, 200 and 400mg/kg/day showed a mean loss of  $1.01 \pm 0.84$ g ( $p = 0.013$ ),

309 1.23±0.86g (P = 0.012), and 0.45±0.66g (P= 0.065) respectively. Mice treated  
 310 with solvent gain a mean body weight of 3.05±0.95g.(See table 1).

311 **Table 1: Body weight of mice in subacute toxicity study of *C. molle* crude**  
 312 **extract**

Treatment group	Body weight (g)					
	Initial weight	After 1 week	After 2 weeks	After 3 weeks	After 4 weeks	Body Wt. dif.
Control	28.44±0.97	31.52±0.72	31.45±0.68	31.47±0.54	31.48±0.62	3.05±0.95
CM100mg/kg	26.46±1.27	28.29±1.66	28.65±1.27	26.42±1.48	25.45±2.04	-1.01±0.84 <sup>al</sup>
CM200mg/kg	27.31±0.68	27.11±1.69	24.76±1.26	26.68±1.76	25.89±1.52	-1.23±0.86 <sup>al</sup>
CM400 mg/kg	27.47±0.84	27.43±1.04	24.60±0.63	26.70±1.18	26.73±0.90	-0.45±0.66

313 Results presented as mean ±SEM; n= 6; CM= *Combretum molle*

314

315 The mean weight of kidney in subacute toxicity test at 100, 200 and  
 316 400mg/kg/day was 0.17±0.02g (P= 0.945), 0.17±0.02g (P=0.945) and 0.13±0.01g  
 317 (P= 0.180) respectively. There was no significant difference compared to negative  
 318 control group (0.18±0.01g). (See table 2)

319 The mean weight of liver in subacute toxicity test at 100, 200 and 400mg/kg/day  
 320 was 1.80±0.09g (P=0.242), 1.65±0.10g (P=0.028) and 1.55±0.10g (P=0.008)  
 321 respectively. At doses 200mg/kg and 400mg/kg produced significant differences  
 322 compared to negative control group (p<0.05 and p<0.01) respectively compared to  
 323 the negative control 2.05±0.08g.(See table 2)

324 **Table 2: Absolute organ weight of mice during sub -acute toxicity study of**  
 325 ***Combretum molle* crude extract**

Treatment group	Weight (g)	
	Kidney	Liver
Control	0.18±0.01	2.05±0.08
CM100mg/kg	0.17±0.02	1.80±0.09
CM200mg/kg	0.17±0.02	1.65±0.10 <sup>a1</sup>
CM400mg/kg	0.13±0.01	1.55±0.10 <sup>a2</sup>

326

327 Results presented as mean ±SEM; n= 6, <sup>a</sup> compared to control, <sup>1</sup>p<0.05, <sup>2</sup>p<0.01

328 CM= *Combretum molle*

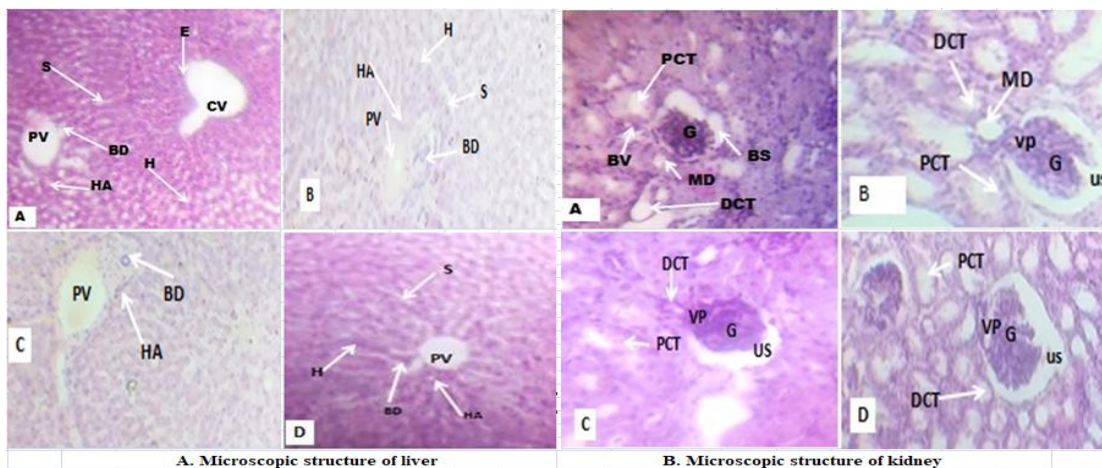
329

330 **Result of histopathology of liver and kidney of mice**

331 **Effects of methanolic leaf extracts of *C. Molle* on histology of the liver and**  
332 **kidney tissues.**

333 Microscopic examination of liver sections of mice treated with *C. molle* leaf crude  
334 extract at 100, 200, and 400mg/kg/day showed the normal architecture of structural  
335 units of the hepatic lobules, formed by cords of hepatocytes separated by hepatic  
336 sinusoids. The central vein and portal area containing branches of hepatic artery,  
337 bile duct and portal veins were maintained with their normal appearance similar to  
338 the negative control (See figure 1A).

339 Microscopic examination of kidney sections of mice treated with *C. molle* leaf  
340 crude extract at 100, 200, and 400mg/kg/day showed the normal architecture of  
341 structural units of the Glomerulus, Urinary space, Proximal convoluted tubule,  
342 Distal convoluted tubule, Macula densa, Vascular pole were maintained with their  
343 normal appearance similar to the negative control (See figure 1B).



344 **A. Microscopic structure of liver** **B. Microscopic structure of kidney**  
345 **Figure 1: Microscopic architectures of liver and kidney after histopathological**  
346 **procedure (A=Negative control, B=100mg/kg, C=200mg/kg, and D=400mg/kg)**  
347 **of *C. molle* crude methanolic extract.**

348 Liver: (CV=Central vein, H=Hepatocytes, E=Endothelial cells, S=sinusoids, K=Kupffer  
349 cells)

350 Kidney: (G =Glomerulus, US=Urinary space, PCT=Proximal convoluted tubule,  
351 DCT=Distal convoluted tubule, MD=Macula densa, VP=Vascular pole)

352 **Antimalarial suppressive test**

353 Antimalarial suppressive test of methanolic crude extract of *C. molle* leaf and  
 354 chloroquine base against *P.berghei* infected mice resulted in reduced parasite load  
 355 as compared to their respective negative control group. Methanolic crude extract at  
 356 100, 200 and 400mg/kg/day and Chloroquine base at 25mg/kg/day showed 33.08,  
 357 45.36 and 76.17 95.71 % parasitemia suppression respectively and the results were  
 358 highly significant( P < 0.001) compared to the negative control group at day-4.(see  
 359 table 3)

360 Methanolic leaf crude extract of *C. molle* leaf at 100, 200, 400mg/kg/day showed  
 361 7.00 ±0.97 (P=0.988), 9.83 ±1.92 (P=0.571), 13.83 ±3.20 (P=0.049) and  
 362 Chloroquine base at 25mg/kg/day showed 27.00 ±2.03 (P<0.001) days of mean  
 363 survival time in four day suppressive test respectively. Chloroquine base at  
 364 25mg/kg/day showed highly significant (P<0.001) in survival days compared to  
 365 negative controls (5.67±0.49)(see table 3).

366 **Table 3: Mean Percent Parasitemia and mean percent parasitemia suppression**  
 367 **and mean survival time of *P. berghei* infected mice treated with methanolic**  
 368 **crude extract of *C. molle* leaf in the 4 day suppressive test.**

Extract	% Parasitemia	% Parasitemia Suppression	Mean survival time
CON	33.40±2.43	0.00	5.67±0.49
CM <sub>100</sub>	22.35 ±1.76	33.08 <sup>a3d3e3</sup>	7.00 ±0.97 <sup>e3</sup>
CM <sub>200</sub>	18.25 ± 2.27	45.36 <sup>a3d1e3</sup>	9.83 ±1.92 <sup>e3</sup>
CM <sub>400</sub>	7.96 ± 2.26	76.17 <sup>a3b3c1</sup>	13.83 ±3.20 <sup>a1e2</sup>
CQ25	1.43 ±0.75	95.71 <sup>a3b3c3</sup>	27.00 ±2.03 <sup>a3b3c3d2</sup>

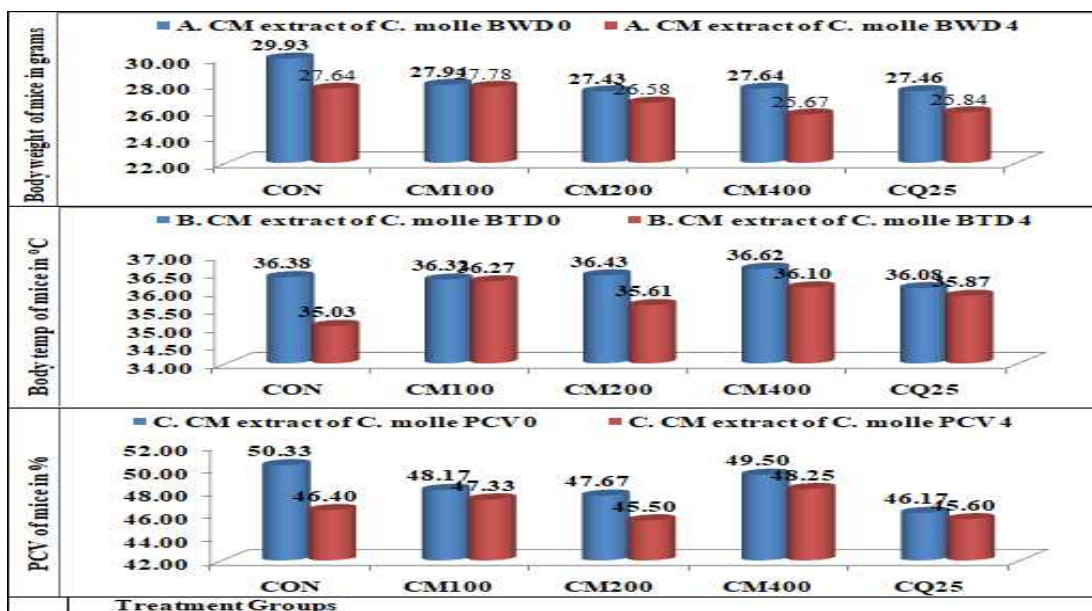
369 Data are expressed as mean ± SEM; n = 6, CON = Control (solvent 10ml/kg), CM=  
 370 crude methanolic extract, CF = Chloroform fraction of *Gardenia ternifolia*, CQ =  
 371 chloroquine, % par supp= % parasitemia suppression, Numbers refer to dose in mg/kg.  
 372 a, compared to neg. control; b, to 100mg/kg; c, to 200mg/kg; d, to 400 mg/kg; e, to  
 373 CQ10mg/kg: 1p<0.05, 2p<0.01, 3p<0.001.

374 *Plasmodium berghei* infected mice treated in a suppressive test at 100mg/kg/day  
 375 methanol crude extract loss mean body weight of 0.85 ±0.210g (p = 0.297),  
 376 200mg/kg/day loss mean body weight of 1.97± 0.673g (P = 0.991), 400mg/kg/day loss  
 377 mean body weight of 1.62±0.825g (P= 0.882), and Chloroquine base at 25mg/kg/day  
 378 loss mean body weight of 0.17 ±0.099g (P= 0.050) and mice treated with solvent loss  
 379 mean body weight of 2.29 ±0.339g. The extract of *C. molle* leaf prevented a loss of

380 body weight in infected mice at high doses. Small decrease in body weight was  
 381 observed in Chloroquine treated group. (See figure 2A)

382 *Plasmodium berghei* infected mice treated in suppressive test at 100mg, 200mg,  
 383 400mg /kg/day methanol crude extract and Chloroquine base at 25mg/kg/day decrease  
 384 a mean body temperature of  $0.82 \pm 0.16$  °C ( $p = 0.106$ ),  $0.52 \pm 0.13$ °C ( $P = 0.003$ ),  
 385  $0.22 \pm 0.17$  °C ( $P < 0.001$ ) and  $0.05 \pm 0.03$  °C ( $P < 0.001$ ) and mice treated with solvent  
 386 decrease a mean body temperature of  $1.35 \pm 0.18$  °C. The extract of *C.molle* leaf  
 387 decreases a mean body temperature in infected mice from 200mg/kg/day ( $P \leq 0.003$ ).  
 388 (See figure 2B)

389 *Plasmodium berghei* infected mice treated in suppressive test at 100mg, 200mg,  
 390 400mg /kg/day methanol crude extract and Chloroquine base at 25mg/kg/day loss a  
 391 mean packed cell volume of  $1.75 \pm 0.63$  % ( $p = 0.795$ ),  $1.25 \pm 0.48$  % ( $P = 0.315$ ),  
 392  $0.80 \pm 0.37$  % ( $P=0.60$ ) and  $0.83 \pm 0.17$  % ( $P= 0.050$ ) and mice treated with solvent loss  
 393 a mean packed cell volume of  $2.40 \pm 0.40$  %. The extract of *C.molle* leaf did not show  
 394 a significant difference in a mean packed cell volume compared to the negative  
 395 control. (See figure 2C)



396 **Figure 2: Showed effect of Crude methanolic extract of *C. molle* leaf in *P. berghei***  
 397 **infected mice in Chemosuppressive test.** [CON = negative control (solvent 10ml/kg),  
 398 CM= chloroform fraction, CQ = chloroquine, BWD0= body weight at day zero,  
 399

400 BWD4= body weight at day 4, PCV= packed cell volume, numbers = dose in  
401 mg/kg/day]

402 Methanolic crude extract in a curative test at 100, 200 and 400mg/kg/day and  
403 Chloroquine base at 25mg/kg/day showed 43.3, 62.98 and 68.48 and 99.05 %  
404 parasitemia suppression respectively and the results were highly significant( P <  
405 0.001) compared to the negative control group at day-7.(see table 4)

406 Methanolic leaf crude extract of *C. molle* leaf at 100, 200, 400mg/kg/day showed  
407 7.83±0.40 (P=0.997), 9.50±0.72 (P=0.248), 12.17±1.01 (P<0.001) and Chloroquine  
408 base at 25mg/kg/day showed 28.83±0.65 (P<0.001) days of mean survival time in  
409 curative test respectively. Crude methanolic extract 400mg/kg and Chloroquine base at  
410 25mg/kg/day showed highly significant (P<0.001) in survival days compared to  
411 negative controls (7.50±0.34)(see table 4).

412 **Table 4: Parasitaemia and percent parasitemia inhibition and survival date of *P.***  
413 ***berghei* infected mice treated with crude methanolic extract of *C.molle* in the**  
414 **Rane's (curative) test**

Extract	%Para Day 3	%Para Day4	%Para Day 5	% Para Day 6	Day 7		Av. Survival date
					%Para	% Parasitemia inhibition	
CON	22.73±0.84	24.95±0.92	27.55±0.73	32.68±0.29	38.76±0.82	0.00	7.50±0.34
CM <sub>100</sub>	24.77±0.72	24.58±0.73	23.72±0.65	22.77±0.67	21.98±0.76	43.3 <sup>a3c3d3e3</sup>	7.83±0.40 <sup>d3e3</sup>
CM <sub>200</sub>	22.33±0.60	23.02±0.48	20.48±0.65	17.37±0.79	14.35±1.01	62.98 <sup>a3b3e3</sup>	9.50±0.72 <sup>c3</sup>
CM <sub>400</sub>	22.57±0.48	23.32±0.50	17.77±0.49	14.03±0.54	12.22±0.49	68.48 <sup>a3b3e3</sup>	12.17±1.01 <sup>a3b3e3</sup>
CQ <sub>10</sub>	23.17±0.84	17.53±0.97	8.07±1.92	2.37±0.90	0.37±0.27	99.05 <sup>a3b3c3d3</sup>	28.83±0.65 <sup>a3b3c3e3</sup>

415 Data are expressed as mean ± SEM; n = 6: CQ = chloroquine, CON = Control, CM,  
416 crude methanolic extract of *Combretum molle*, % par supp= % parasitemia  
417 suppression, Numbers refer to dose in mg/kg. a, compared to neg. control; b, to  
418 100mg/kg; c, to 200mg/kg; d, to 400 mg/kg; e, to CQ 25mg/kg: 1p<0.05, 2p<0.01,  
419 3p<0.001.

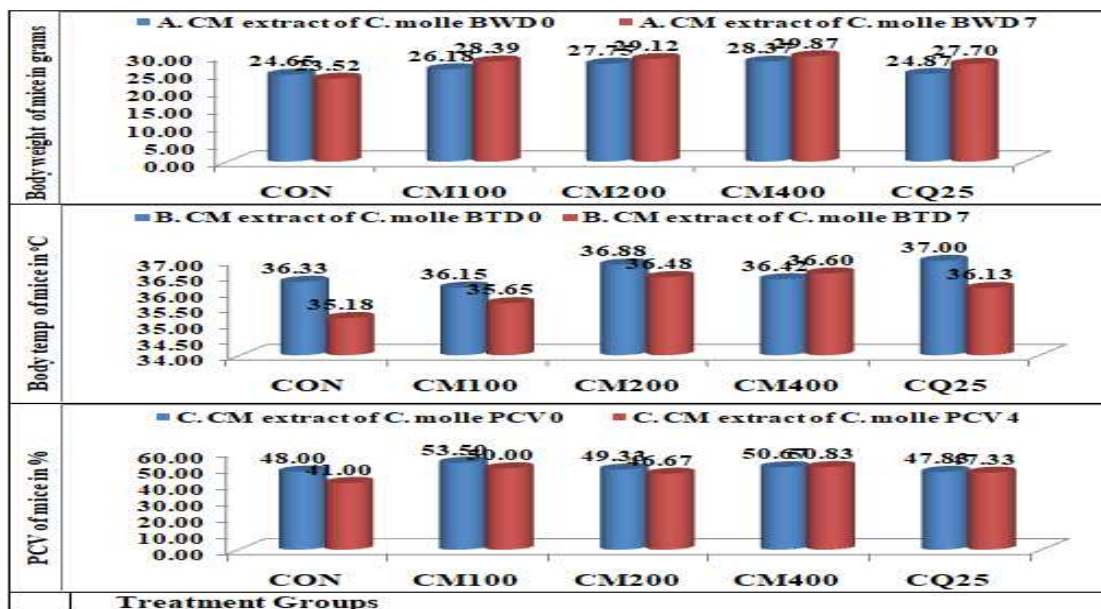
420 *Plasmodium berghei* infected mice treated in a curative test at 100mg/kg/day methanol  
421 crude extract increase a mean body weight of 2.41±1.912g (p = 0.011), 200mg/kg/day  
422 loss mean body weight of 0.88±2.140g (P = 0.128), 400mg/kg/day loss mean body  
423 weight of 4.38±1.014g (P= 0.697), and Chloroquine base at 25mg/kg/day loss mean  
424 body weight of 2.42±0.927g (P= 0.315) and mice treated with solvent loss mean body  
425 weight of 8.18±3.624g. (See figure 3A)

426 *Plasmodium berghei* infected mice treated in a curative test at 100mg, 200mg, 400mg  
427 /kg/day methanol crude extract and Chloroquine base at 25mg/kg/day decrease a mean



428 body temperature of  $0.95 \pm 1.36$  °C ( $p = 0.673$ ),  $0.73 \pm 1.05$  °C ( $P = 0.991$ ),  $1.01 \pm 1.05$  °C  
 429 ( $P = 0.998$ ) and  $4.86 \pm 1.48$  °C ( $P < 0.001$ ) and mice treated with solvent decrease a mean  
 430 body temperature of  $1.57 \pm 1.63$  °C. (See figure 3B)

431 *Plasmodium berghei* infected mice treated in a curative test at 100mg, 200mg, 400mg  
 432 /kg/day methanol crude extract and Chloroquine base at 25mg/kg/day loss a mean  
 433 packed cell volume of  $6.65 \pm 1.89\%$  ( $p = 0.001$ ),  $5.41 \pm 0.84\%$  ( $P < 0.001$ ),  $0.53 \pm 1.24\%$   
 434 ( $P < 0.001$ ) and  $1.02 \pm 0.45\%$  ( $P < 0.001$ ) and mice treated with solvent loss a mean  
 435 packed cell volume of  $15.02 \pm 1.04\%$ . The extract of *C.molle* leaf showed a significant  
 436 difference in a mean packed cell volume compared to the negative control. (See figure  
 437 3C)



438 **Figure 3: Showed effect of Crude methanolic extract of *C. molle* leaf in *P. berghei***  
 439 **infected mice in Curative test.** [CON = negative control (solvent 10ml/kg), CM=  
 440 chloroform fraction, CQ = chloroquine, BWD0= body weight at day zero, BWD7=  
 441 body weight at day 7, PCV= packed cell volume, numbers = dose in mg/kg/day]

442 Antimalarial repository test of methanolic crude extract of *C. molle* leaf and  
 443 chloroquine base against *P.berghei* infected mice resulted in reduced parasite load as  
 444 compared to their respective negative control group. Methanolic crude extract at 100,  
 445 200 and 400mg/kg/day and Chloroquine base at 25mg/kg/day showed 51.00, 62.65,  
 446 76.20 and 99.20% parasitemia suppression respectively and the results were highly  
 447 significant( $P < 0.001$ ) compared to the negative control group at day-7.(see table 5)  
 448

449 Methanolic leaf crude extract of *C. molle* leaf at 100, 200, 400mg/kg/day in repository  
 450 test showed 7.50±0.99 (P=0.977), 10.00±2.31 (P=0.550), 17.67±3.05 (P=0.001) and  
 451 Chloroquine base at 25mg/kg/day showed 29.00±0.63 (P<0.001) days of mean  
 452 survival time in seven-day repository test respectively. Chloroquine base at  
 453 25mg/kg/day showed highly significant (P<0.001) in survival days compared to  
 454 negative controls 6.00±0.97 (see table 5).

455 **Table 5: Mean Percent Parasitemia and mean percent parasitemia suppression**  
 456 **and mean survival time of *P. berghei* infected mice treated with methanolic crude**  
 457 **extract of *C. molle* leaf in repository (prophylactic) test.**

Extract	% Parasitemia	% Parasitemia Suppression	Mean survival time
CON	24.90±2.16	0.00	6.00±0.97
CM <sub>100</sub>	12.20±0.41	51.00 <sup>a3d2e3</sup>	7.50±0.99 <sup>d2e3</sup>
CM <sub>200</sub>	9.30±0.56	62.65 <sup>a3e3</sup>	10.00±2.31 <sup>d1e3</sup>
CM <sub>400</sub>	5.93±0.49	76.20 <sup>a3b2e2</sup>	17.67±3.05 <sup>a2b2c1e3</sup>
CQ25	0.20±0.20	99.20 <sup>a3b3c3d2</sup>	29.00±0.63 <sup>a3b3c3d3</sup>

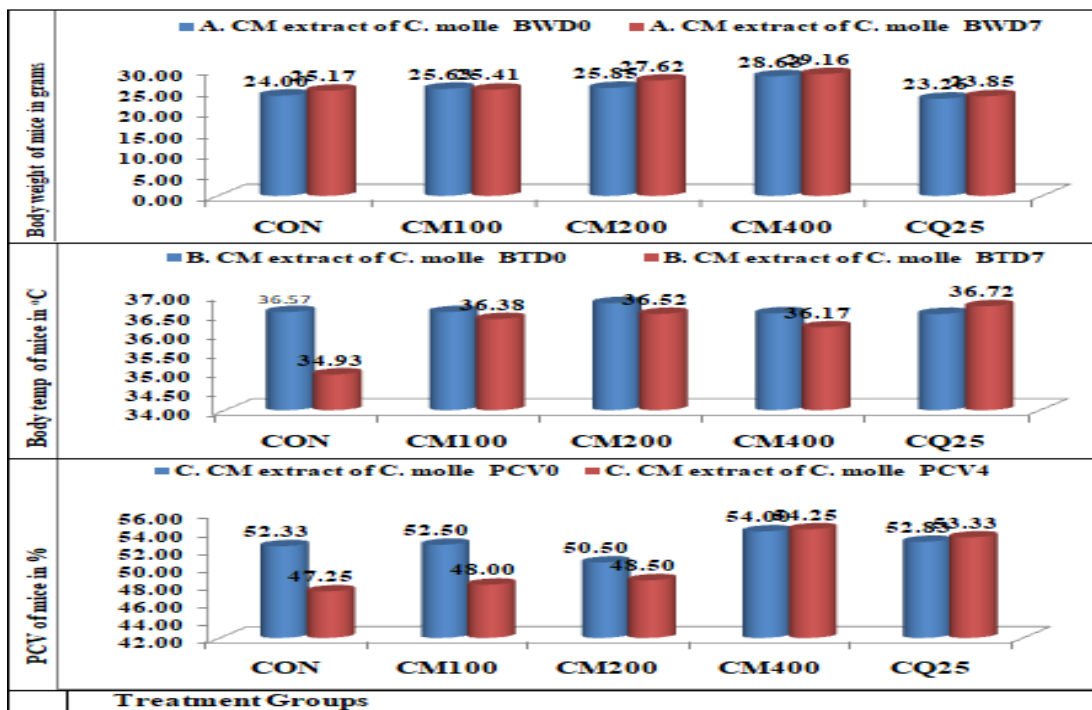
458 Data are expressed as mean ± SEM; n = 6: CQ = chloroquine, CON = Control, CM,  
 459 crude methanolic extract of *Combretum molle*, % par supp= % parasitemia  
 460 suppression, Numbers refer to dose in mg/kg. a, compared to neg. control; b, to  
 461 100mg/kg; c, to 200mg/kg; d, to 400 mg/kg; e, to CQ 25mg/kg: 1p<0.05, 2p<0.01,  
 462 3p<0.001.

463 *Plasmodium berghei* infected mice treated in a repository test at 100mg/kg/day  
 464 methanol crude extract of *C. molle* increase a mean body weight of 0.37±1.39g (p =  
 465 1.000), 200mg/kg/day increase mean body weight of 1.84±1.08g (P = 0.665),  
 466 400mg/kg/day increase mean body weight of 0.56±0.67g (P= 0.988), and Chloroquine  
 467 base at 25mg/kg/day increase mean body weight of 0.08±0.93g (P = 1.000) and mice  
 468 treated with solvent loss mean body weight of 0.15±1.01g. (See figure 4A)

469 *Plasmodium berghei* infected mice treated in a repository at 100mg, 200mg, 400mg  
 470 /kg/day methanol crude extract of *C. molle* and Chloroquine base at 25mg/kg/day  
 471 increase a mean body temperature of 0.75±0.30°C (p = 0.009), 0.27±0.41°C (P =  
 472 0.149), 0.62±0.12°C (P< 0.021) and 0.75±0.16°C (P=0.009) and mice treated with  
 473 solvent decrease a mean body temperature of 0.67±0.27°C. (See figure 4B)

474 *Plasmodium berghei* infected mice treated in a repository at 100mg, 200mg, 400mg  
 475 /kg/day methanol crude extract of *C. molle* decrease a mean packed cell volume of  
 476 3.25±0.95% (p = 0.998), 1.25±0.85% (P =0.984) and 9.40±12.23%, (P=0.995)

477 respectively and Chloroquine base at 25mg/kg/day decrease a mean packed cell  
 478 volume of  $0.50 \pm 0.56\%$  ( $P=0.934$ ) and mice treated with solvent increase a mean  
 479 packed cell volume of  $6.00 \pm 0.41\%$ . (See figure 4C)



480  
 481 **Figure 4: Showed effect of Crude methanolic extract of *C. molle* leaf in *P. berghei***  
 482 **infected mice in prophylactic test.** [CON = negative control (solvent 10ml/kg), CM=  
 483 chloroform fraction, CQ = chloroquine, BWD0= body weight at day zero, BWD7=  
 484 body weight at day 7, PCV= packed cell volume, numbers = dose in mg/kg/day]

485 Discussion

486 The rodent malaria model in mice is the most extensively used, and ideal for the  
 487 primary *in vivo* tests of new antimalarials. Mice are easy to raise, maintain, handle,  
 488 and are small (20-30g), thus requiring a small amount of drugs compared to chickens,  
 489 rats or monkeys. Mice are also susceptible to several species of malaria parasites  
 490 isolated in other rodent species. Mice may develop acute fulminating diseases induced  
 491 by blood-stage parasites, or by sporozoites injected either by mosquito bites or  
 492 intravenously, being excellent models for testing new drugs.<sup>[11]</sup>

493 The leaf of *C. molle* methanolic crude extract phytochemical screening showed the  
 494 presence of secondary metabolites: alkaloids, Cardiac glycosides, flavonoids, phenols,  
 495 saponnins, tannins and terpenoids by using specific chemicals and methods <sup>[23]</sup>. These

496 compounds are known to show curative activity against several ailments in man and  
497 therefore could explain the traditional use of medicinal plants for the treatment of  
498 some illnesses [29, 30, 31].

499 Crude Methanolic extract was non-toxic to test mice, as they did not show signs of  
500 acute toxicity for 14 days at the doses of 2000mg/kg body weight, thus the traditional  
501 use of the plant material by local people like the decoction of root, leaf, and fruit could  
502 be safe provided that the amount used is in the ranges tested in this study. However,  
503 species variation would also limit such a straight forward extrapolation of the findings  
504 of this study to humans.

505 The subacute toxicity evaluation of *C. molle* crude methanolic leaf extract showed a  
506 mean body weight decrease from week 0 to week 4 in a decreasing order from  
507 100mg/kg/day to 400mg/kg/day and mice treated with the solvent showed a mean  
508 weight gain from week 0 to week 4.

509 The mean weight of kidneys of mice treated with the three different doses of *C.molle*  
510 methanolic crude leaf extract in subacute toxicity evaluation did not show any  
511 significant difference compared to the negative control.

512 The mean weight of liver of mice treated with the three different doses of *C.molle*  
513 methanolic crude leaf extract in subacute toxicity evaluation showed significant  
514 differences at 200mg/kg/day and 400mg/kg/day compared to the negative control.

515 The histopathological examination of liver and kidney tissues in subacute toxicity of  
516 mice treated with 100, 200, and 400mg/kg/day body weight of *C. Molle* crude  
517 methanolic leaf extract showed normal histological structures similar to the negative  
518 control group.

519 The crude methanolic extract of dried stem of *C. molle* in *In vitro* cell culture with  
520 *plasmodium falciparum* also showed an activity with IC<sub>50</sub> value of 1.25 µg/ML [20, 21].

521 So the methanolic crude extract obtained from *C. molle* showed high *in vitro* activity  
522 which helps to proceed *in vivo* study. Therefore, the present study was carried out on  
523 *in vivo* antimalarial study of the methanolic crude extract of *C. molle* leaf in  
524 *Plasmodium berghei* infected mice. Methanol crude extract showed dose-dependent  
525 activity against *plasmodium berghei* in a 4-day suppressive test. In comparing the  
526 result obtained from the present study the three doses of crude methanolic extract

527 showed significant parasitemia suppression compared to the negative control. *In vivo*  
528 antiplasmodial activity can be classified as moderate, good, and very good if an extract  
529 displayed percent parasite suppression equal to or greater than 50% at a dose of 500,  
530 250 and 100mg/kg body weight per day respectively <sup>[12]</sup>. Based on this classification,  
531 the methanolic crude extract at 200 and 400 mg/kg/day showed 45.36% and 76.17 %  
532 suppression against *P. berghei* in mice respectively and exhibited good to moderate  
533 antiplasmodial activity. These result indicated that compounds which have  
534 antimalarial activity were soluble in the solvent used to extract.

535 The crude methanolic extract of *C.molle* in a four day suppressive test at all doses did  
536 not produce significant difference in body weight and packed cell volume compared to  
537 the negative control but they produce significant difference (P< 0.003) from  
538 200mg/kg/day in reducing body temperature. The crude methanolic extract at doses of  
539 100, 200 and 400mg/kg/day showed increased mean survival time compared to the  
540 negative control but didn't produce a significant difference from the negative control.  
541 Mice treated with chloroquine base 25mg/kg/day showed a significant difference  
542 compared to the negative control.

543 In the curative test the average percent parasitemia was increased in the negative  
544 control from 3<sup>rd</sup> day to the 7<sup>th</sup> day. The *Plasmodium berghei* infected mice treated  
545 with crude extract the average percent parasitemia increased from the day -3 to day -4  
546 and showed a decrease in percent parasitemia from the day-5 to day -7 in a dose-  
547 dependent manner compared to the negative control. The infected mice treated with  
548 Chloroquine base showed a decrease in average percent parasitemia from 3<sup>rd</sup> day to the  
549 7<sup>th</sup> day.

550 In the curative test crude methanolic extract of *C. molle* produced significant  
551 difference in percent packed cell volume compared to the negative control. The crude  
552 methanolic extract at doses of 100, 200 and 400mg/kg/day showed increased mean  
553 survival time compared to the negative control and but only produced significant  
554 difference at 400mg/kg/day compared to negative control.

555 Methanolic crude extract in repository(prophylactic) test at 100, 200 and  
556 400mg/kg/day and Chloroquine bate at 25mg/kg/day showed 51.00, 62.65, 76.20 and

557 99.20% parasitemia suppression respectively and the results were highly significant( P  
558 < 0.001) compared to the negative control group at day-7.

559 The promising result from the curative and prophylactic tests suggests that the crude  
560 extract has therapeutic efficacy against established malarial parasitic infections. The  
561 observed significant percent parasitemia inhibition, prevention of body weight  
562 reduction, increase in packed cell volume, body temperature and mean survival time in  
563 four-day suppressive test and observed curative potential in established *Plasmodium*  
564 *bergei* infection were expected to be from the effects of the secondary metabolites  
565 such as Inhibition of extracellular microbial enzymes, deprivation of the substrates  
566 required for microbial growth or direct action on microbial metabolism through  
567 inhibition of oxidative phosphorylation<sup>[25]</sup>. Intercalation of DNA and inhibition of  
568 DNA synthesis through topoisomerase inhibition and termination of cell division,  
569 complexation with extracellular and soluble proteins and disruption of microbial  
570 membranes, enzymes, hydrogen bonding and hydrophobic effects, as well as by  
571 covalent bond formation, inactivation of microbial adhesins, enzymes, cell envelope  
572 transport proteins<sup>[27]</sup>. Inhibiting growth, and protease activity <sup>[29]</sup>. Capable of  
573 precipitating gelatinous compounds, phagocytic cells, host-mediated tumor activity  
574 <sup>[30]</sup>.

### 575 **Conclusion**

576 The present study indicates that 80% methanolic crude extract of *Combretum molle*  
577 leaf has good antimalarial on the measured parameters. The crude methanolic extract  
578 showed significant parasitemia suppression and mean survival time in the 4-day  
579 Chemosuppressive, curative and prophylaxis tests but other parameters such as  
580 prevention in a reduction of body weight, body temperature, in protecting infected  
581 animals from parasite-induced PCV reduction were not significantly different  
582 compared to the negative control. The subacute toxicity evaluations showed no  
583 toxicity at the three different doses in histopathological examination. Findings suggest  
584 that the phytochemicals responsible for antimalarial activity of the plant are soluble in  
585 80% methanol which upholds the earlier in vitro findings as well as the folkloric use in  
586 Ethiopian traditional medicine.

### 587 **Recommendation**

588 We forward the recommendation for investigators to carry out in-depth study to  
589 isolate, identify and characterize the bioactive constituents responsible for antimalarial  
590 activity of leaf of the *C.molle*. In the present study acute and sub-acute toxicity was  
591 done showing that the plant was safe. Therefore, we recommend performing chronic  
592 histopathological and biochemical toxicity study of the plant.

593 **Ethical approval:**

594 The animals were handled according to the governance of animal care and use for  
595 scientific purposes in Africa and the Middle East [33]. And the ethical clearance was  
596 requested and obtained from the Department of Pharmacology, School of  
597 Pharmacy, University of Gondar for the use of animals for experiment with Ref.  
598 No. 5094/96/2011. And the research was performed as per the agreement.

599 **List of abbreviations:** Not applicable

600 **Consent to publish:** Not applicable

601 **Availability of data and material:** All data generated or analyzed during this study  
602 have been attached on the supplementary material.

603 **Competing interests**

604 The authors declare that they have no competing interest to disclose.

605 **Funding:** No source of funding

606 **Authors' contributions**

607 MA was involved in developing the proposal and designing of the study. MA, GM, TJ,  
608 and EF conducted the actual study and the statistical analysis and in the write up of the  
609 manuscript was done by MA and TJ. All authors gave the final approval of the version  
610 to be published, and agree to be accountable for all aspects of the work.

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615 free delivery of *P. berghei* parasite.

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